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(54) Title: AGROBACTERIUM MEDIATED TRANSFORMATION OF GERMINATING PLANT SEEDS

(57) Abstract

A non-tissue culture process using *Agrobacterium*-mediated vectors to produce transgenic plants from seeds of such plants as the common bean and soybean.

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AGROBACTERIUM MEDIATED TRANSFORMATION OF GERMINATING PLANT SEEDS

FIELD OF INVENTION

This invention relates to a process for transforming th germinating seed of a plant and the use of said process to produc transformed plants, particularly dicotyledonous plants.

BACKGROUND OF THE INVENTION

The development of single gene transfer techniques for plant species is of great interest and value to plant breeders because it can be used for the rapid transfer of beneficial genetic traits t plants. Numerous methods have been developed for transferring gen s into plant tissues; Agrobacterium-mediated transfer (Murai et al., 1983; Fraley et al., 1983), direct DNA uptake (Paszkowski et al., 1984; Potrykus et al., 1985), microinjection (Crossway et al., 1986), high-velocity microprojectiles (Klein et al., 1987) and electrop r-ation (Fromm et al., 1985; Fromm et al., 1986). A general problem with most of these gene transfer techniques is that the transformed tissues, either leaf pieces or cellular protoplast, must be subjected to some regeneration steps which require a considerable amount f time before a whole plant can be obtained. This process is further complicated because tissue culture procedures have not been estab-lished for many crop species. In most cases, gene transfers into crop species have been limited to transformed callus, not whole cr p plants. In addition, tissue culture procedures can result in rearrangement of the inserted DNA; or somatic mutations may occur and result in the loss or alteration of desirable genetic traits accumu-lated by the expertise of many years of plant breeding.

Agrobacterium-mediated gene transfers are by far the most widely used gene transfer techniques, but the use of Agrobacterium strains may be limited because they do not efficiently infect mon c tyl-edonous cereal crop species. However, recent reports (Hooykaas-Van Slogteren et al., 1984; Hernalsteens et al., 1984; Grav s and G ldman, 1986; Grimsley t al., 1987; Schafer et al., 1987; Byt bier t al., 1987) suggest that c nditions xist wher by Agrobacterium strains can bind to m n tyled n us plant cells and transf r their T-DNA r gions int these cells. Int restingly, th rep rt by Graves and Goldman (1986) sugg sts that Agrobacteria can inf ct scut llar and m so otyl cells f germinating corn (Zea mays) s eds and that th

resulting plants are transformed, although these transformed plants will be selected. This technique suggests that Agrobacterium-mediated gene transfer can be accomplished without the need of any tissue culture intermediate steps. Additional support for the transformation of mesocotyl cells of germinating seeds was obtained by Feldmann and Marks (1987) as they were able to obtain G418 resistant Arabidopsis thaliana plants by co-cultivating germinating seeds with Agrobacteria containing a binary plasmid with a plant expressible neomycin phosphotransferase (NPT) II gene in its T-DNA region.

The development of gene transfer techniques for leguminous plants is of commercial interest because it facilitates the development of new cultivars with improved disease resistance, tolerance to specific herbicides and increased nutritional value. Unfortunately, even though these dicotyledonous species are susceptible to Agrobacterium infections (Facciotti et al., 1985; Owens and Cress, 1985; Byrne et al., 1987), its use for transformation is limited due to the lack of available and efficient regeneration procedures, especially for transformed tissues.

Extension of this technique to germinating seed of leguminous plants such as Phaseolus vulgaris, the common bean, is of great importance because regeneration procedures are not available, let alone the regeneration of transformed undifferentiated tissues.

The development of simple, non-tissue culture dependent methods for transfer, stable integration, and sexual transmission of genetic material into plant species is of great interest and importance. Reports from Graves and Goldman (1986) and Feldmann and Marks (1987) present evidence that transformed whole plants can be obtained via Agrobacterium-mediated transformation of the mesocotyl cells of germinating seeds.

The process of this invention represents (1) an improvement of the Graves and Goldman (1986) technique for the transformation of the seeds of monocotyledous plants and (2) its extension to dicotyledonous plants.

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A non-tissue culture approach for preparing transformed Arabidopsis thaliana seeds is described by Feldmann and Marks, Mol. Gen. Genet. (1987) 208:19. However, to the inventors' knowledge the application of non-tissue culture transfer has not been successfully applied to leguminous plants and other large seed dicots such as soybean, the common bean, squash, zucchini, peppers, and others.

SUMMARY OF THE INVENTION

The present invention provides:

A process for producing a transgenic plant which comprises:

(a) germinating a seed of a plant;

(b) inoculating the meristematic or mesocotyl cells pr duc d during germinati n, pri r to their diff rentiati n, with a virulent r non-virul nt Agrobacterium strain containing a transf rable gene in an Agrobacterium deriv d vector; and

(c) all wing th c lls t diff rentiate into matur plants, with th provis that th plant cannot be fr m the family Arabidopsis thaliana.

The time of infecting germinating P. vulgaris seed after germination with the Agrobacterium-based vectors has been found to be critical. The length of time the seeds are allowed to germinate prior to Agrobacteria infection will greatly affect the ability of the Agrobacteria to infect meristematic cells, because the amount of vascular tissue is rapidly increasing as differentiation proceeds. However, seed germination must take place in order to have physical access to the mesocotyl region. Therefore a preferred manner of practicing the invention is to conduct the inoculation step within 16 to 96, preferably 24 to 48, hours of germination. To determine the optimum time for infecting germinating seeds, inoculations with virulent Agrobacterium strain A208, were done at various times after initiating germination, between 6 to 96 hours. Successful transformation was scored by gall formation on the developing seedlings, the results of inoculating 50 seeds for each time interval is presented in Table I. Seeds allowed to germinate between 24 to 48 hours were found to be the most susceptible to Agrobacterium infections. Between 70% to 80% of these inoculated seeds gave rise to seedlings with galls formed either on the hypocotyl, epicotyl, cotyledonary node, or distributed throughout the base of the plant. A preferred method of inoculation is with a virulent or non-virulent Agrobacterium strain containing a transferable DNA cis or trans plasmid

A particularly preferred manner of practicing the process on dicots involves removing one of the cotyledons prior to inoculation. This step increases access of the strain to the mesocotyl region wherein the meristematic cells are generated.

The method of this invention is simple, rapid, avoids the use of any tissue culture techniques, and transformed plants can be obtained directly.

Also provided are:

Transgenic plants prepared by the process of this invention. Preferred are dicotyledonous transgenic plants. Especially preferred are dicotyldonous plants of the family leguminoseae, such as phaseolus vulgaris and Glycinus max.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Germinating seeds are inoculated with either virulent or non-virulent Agrobacterium tumefacien or Agrobacterium rhizogenes strains which contain the binary plasmid pGA472 or PGA482 or their deriv-

atives. Both ar availabl fr m Dr. G. An, Washingt n State University, Pullman, WA. This binary plasmid nc des a plant expr ssible NPT II gene within its T-DNA region and their derivativ s c ntain genes that will convey useful traits to transformed species. M st plants resulting from seeds inoculated with virulent Agrobacterium strains, which also contained the binary plasmid, developed typical crown galls. However, NPT II activity was found in the leaves f some inoculated whole plants, indicating that the binary T-DNA r gi n was also transferred. Transfer of the binary T-DNA region was als accomplished by using avirulent strains of A. tumefaciens or rhizogenes. Results presented here show that 1.6% of the P. vulgaris and about 1% of the Glycine Max (soybean) plants were transformed, with transformation being determined by the presence of NPT II enzym activity.

Seeds of Phaseolus vulgaris cv. Olathe or Glycine max (cV.A0949) were surface sterilized with 15% Clorox for 10 minutes, followed by 4-5 rinses with distilled water and then placed on moistened paper towels in a temperature controlled Percival incubator at 28°C. and allowed to germinate for various times, 16 to 96 hours. Seed c ats were removed and the decoated seeds were opened in halves (that is how cotyledons were removed from the main seed body). The mesoc tyl region of the germinating seeds, with their plummule still attach d, were infected with an overnight liquid culture of various Agrobacterium strains by using an Eppendorf pipetter fitted with a 27 1/2 gauge needle. Seeds were infected with virulent or avirulent A. tumefaciens strains (A208, C58, C58z707 and A208/phas-zein) or A. rhizogenes strains [A4RS and A4RS(pR:B278b)pu3.3c-1]. The common A. tumefaciens and A. rhizogenes strains are available from ATCC, 12301 Parklawn Drive, Rockville, MD. The disarmed A. rhizogenes strain RS(pRiB278b) has been described by Vilaine and Casse-Delbart (1987) Mol. Gen. Genet., 206,17 and is available from Dr. F. Casse-Delbart, C.N.R.A., Routede Saint Cyr, F78000, Versailles, France. The disarmed A. tumefaciens, strain C582707 is availabl fr m Dr. A. G. Hepburn, Univ rsity f Illinois, Urbana, IL. Inoculated s ds wer then placed n m ist n d paper towels in petri dishes and incubated at 28°C. After f ur days thes s dlings wer transformed to soil and gr wn t maturity in th gr enh us . Plants infected with virulent strains f A. tumefaciens wer sc r d for fficiency f gall

formation as a function of germination time.

NPT II Enzyme Activity

NPT II enzyme activity was detected by the in situ gel assay as reported by Reiss et al. (1984). Briefly, 100 mg. of a leaf tissue was mixed with 20 ml. of extraction buffer in a 1.5 ml. Eppendorf tube. Tissue samples were macerated with a Konte pestle and centrifuged for 20 minutes at 4°C. A 35 µl aliquot of the supernatant solutions was electrophoresed on a non-denaturing 10% polyacrylamide gel. The gel was overlaid with a 1% agarose gel containing 67 mM. tris-maleate (pH 7.1), 42 mM. $MgCl_2$, 400 mM NH_4Cl, 20 µg kanamycin sulfate and 200 µCi gamma-[^{32}P]ATP. After incubating for 30 minutes at room temperature, the agarose gel was blotted onto Whatman P81 phosphocellulose paper overnight. The P81 paper was removed, washed several times with hot water (80°C.) and autoradiographed.

The following examples utilize many techniques well known and accessible to those skilled in the arts of molecular biology and manipulation of Agrobacterium strains and plasmids (virulent, avirulent, cis- or trans- configurations). Enzymes are obtained from commercial sources and are used according to the vendor's recommendations or other variations known to the art. Reagents, buffers and culture conditions are also known to those in the art. General references containing such standard techniques include the following: R. Wu, ed. (1979) Meth. Enzymol. Vol. 68; J. H. Miller (1972) Experiments in Molecular Genetics; T. Maniatis et al. (1982) Molecular Cloning; A Laboratory Manual; and D. M. Glover, ed. (1985) DNA Cloning Vol. II, all of which are incorporated by reference.

The purpose of these examples is to show that gene constructions exist, either constructed by us or others, which when transferred, integrated, and expressed in a plant will convey a useful trait to that plant.

Example 1

Germinating P. vulgaris and G. max seeds were inoculated about 24 hours after germination with virulent and avirulent Agrobacterium strains which contained a difid pGA482G [constructed by clearing the SalF fragment from pWP866 which contains the gene for gentamycin-(3)-N-acetyl-bnferose III, and is available from W. Pippersberg, P-8080, Munich, Federal Republic of Germany, into one of the SalI sites in pGA482, based binary vector constructions pPhas-z in [which contains

th rn beta-z in gene (P ders n t al., 1987 and is availabl from Dr. B. Larkins, Purdue University, West Lafay tte, IN) transcripti n-ally linked to the P. vulgaris seed st rage prot in gen pr m tor (Slightom et al., 1983) or pu3.3c-1 [which contains the phas lin minigene construction (Chee et al., 1985) and is available fr m Agrigenetics Corp, Madison, WI]. Physical maps of these binary plasmids are presented in Chart 2.

Transfer and expression of the plant expressible NPT II gene contained within the T-DNA region of pGA482G (An et al., 1984) was determined by removing two to three young leaves (usually obtain d 10 inches or more above the wound site resulting from inoculating the germinating seeds), extracting the soluble proteins and testing f r NPT II activity. From a total of 695 plants tested only 11 plants showed NPT II activity in these protein extracts. They are list d in Table II and the NPT II positive results are shown in Chart 2. Ab ut 1.6% of the surviving inoculated seeds show NPT II activity, sugg st-ing that the T-DNA region of the binary plasmid pGA482G is integrat d in the genome of these P. vulgaris plants.

Other procedures, well known to those skilled in the art, such as microinjection and high-velocity microprojectiles, can be used t transfer DNAs into the mesocotyl region and that transformed plants should result.

TABLE I

Frequency of Gall F rmation on Seedlings Inoculated With the Agrobacterium Strain A208

Germination Periods	Frequency of Gall Formation
6 hours	0
12 hours	0
24 hours	80
36 hours	70
48 hours	40
72 hours	10
90 hours	10

TABLE II

NPT II Positive Transformed Plants

Plant Number	Binary Construction	Gall
40	C58/phas-zein	+
41	C58/phas-zein	+
46	C58/phas-zein	+
61	C58/phas-zein	-
65	C58/phas-zein	-
151	C58/phas-zein	+
258	A4RS(pR:B278b)pu3.3c-1	-
269	A4RS(pR:B278b)pu3.3c-1	-
296	A4RS(PR:B278b)pu3.3c-1	-
470	A208/phas-zein	-
552	C58Z707/phas-zein	-

Example 2

Construction f a micro-Ti plasmid for the expressi n of a phas olin mini-gen . Th transfer and xpressi n f this g n will increase the level of seed storage pr tein in the transformed plant.

2.1

Using the P. vulgaris seed storage protein gene, phaseolin, and its cDNA counterpart a mutant phaseolin gene lacking its five introns was constructed. This mutant phaseolin gene (phas-minigene) retains it natural 5' and 3' plant-regulatory sequences and the constructi n of this plasmid (pPv3.3-cDNA) has been described by Chee et al. (1986) Gene 41:47 and Cramer et al. (1985) Proc. Natl. Acad. Sci. 82;334 and is available from Agrigenetics Corp. Madison, WI. Plasmid pPv3.3-cDNA was subjected to restriction enzyme digests, BamHI and HindIII and a 3.6 kb fragment was removed and cloned into BglII and HindIII sites of the binary vector pGA482 (An et al. (1985) EMBO. J. 4:277). This construction places this mutant phaseolin gene within the right and left borders of the binary plasmid, now referred t as pμ3.3c-1, and along side of the plant expressible NPT II gene which is used for selection and identification of transformed plants. The structure of binary plasmid pu3.3c-1 is shown in Chart 1.

2.2 Use of pu3.3c-1

This binary plasmid has to be transferred into various Agrobacterium strains, i.e. A208, C58, C58:707, LBA4404 and A4RS, tc. The method described here can be used to transfer the binary plasmid pμ3.3c-1 into various plant species (e.g., common bean, soybean and other large seeded plants). In addition, multiple copies of the phaseolin minigene can be placed into the binary plasmid by subcloning the NcoI to BamHI fragment (3 kb fragment) frompPv3.3-CDNA into NcoInd BamHI digested clone pPr 8.8 g (available from J. Slightom, The Upjohn Company, Kalamazoo, MI) which replaces th genomic part with the CDNA region of pPV3.3-cDNA. This cl ning experiment results in obtaining subclone pPv8.3-cDNA which contains an upstr am BglII site (Slightom, et al. (1983) Proc. Natl. Acad. Sci., 80:1897) which allows for the is lati n of a BglII-BamHI 3.3,5 kb fragm nt which was recl ned into th BamHI dig sted plasmid pPv3.3-cDNA. The ori ntati n of the new phas lin insert(s) can b checked and nly those in the 5' and 3' ri ntation with respect to the first phaseolin gen ar us d f r additi nal ins rtions. Becaus

only th 3' BamHI site was r tained (the BglII/BamHI ligated sit is not digestible by eith r enzyme) this step could be r peated any number f times, dep nding n plasmid stability and ability t still transform E.coli and Agrobacteria. This procedure was repeated to obtain as many as four phaseolin gene inserts, which were clon d using a HindIII and BamHI digest into the binary plasmid pGA482G. Having a series of these plasmids with different numbers of phas lin genes (this can also be referred to as gene family transfer since a family of similar genes is transferred in a single event) will increase the level of storage proteins in seeds of transform d plants.

Example 3

The purpose of this example is to incorporate a modified se d storage protein which encodes a higher percentage of sulfur-containing amino acids; such a gene is referred to as High Sulfur St rag Protein (HSSP)-gene. This gene is constructed so that it is devel p-mentally expressed in the seeds of dicotyledonous plants; this has been accomplished by using the phaseolin promoter. The modified g n must encode a substantial number of sulfur-containing amino acids. Naturally occurring HSSP-genes can also be used. The two best naturally occurring HSSP-genes are the beta zein gene (15 kD) (P d r-sen et al (1986) J. Biol. Chem. 201:6279) and the Brazil nut protein (Altenbach et al. (1987) Plant Mol. Bio. 8:239). However, any th r natural or synthetic gene derivative of an HSSP-gene can be used f r the improvement of the nutritional value of seeds.

3.1 Construction of a HSSP-gene

The construction of the zein derivative HSSP-gene uses th phaseolin gene promoter from clone pPv8.8-Bg [constructed by doing sight specific modification of pPv8.8g. The BglII to XbaI fragm nt for pPV8.8g was cloned into M13mp 17 (commercially availabl) t obtain clone as 13mp18PV1.6. This was then used to produce singl - stranded DNA which was annealed to an oligomer (30 residues) which c ntain d a two-base pair chang from the riginal phaseolin prom ter r gi n. Th s quenc f th ligomer was 5'CATCATAGTAGATCTAGTATT-GAATATGAG-3'(pposit t oding strain). Aft r annealing DNA polymeras I (Klenow fragment) was added and the remaining pposite strand f M13MP18pv1.6 was synth siz d. Th mutant M13 cl n , containing a new Bgl site 7 bp from the translation start site

(Slight m et al, 1983, ibid) f th phaseloin gen , was scr ened using th 32p-label d olig m r and differ ntial temperature hybridization. Cl ned candidates w re further analyz d by doing Bgl II digestions and agarose gel electroph resis to identify particular clones containing the extra Bgl II site, the appearance of the Bgl II to Bgl II 800 bp fragment. The modified clone ml3 mp181.6 30.12.3 was isolated and DNA was isolated. From the isolated DNA an NcoI to XbaI fragment was removed and cloned into NcoI and the partial XbaI digested p 8.8g. The new clone containing the phaseolin promoter n a 800 bp Bgl II to Bgl II fragment was designated p Pv8.8g Bg.] t ensure proper expression and at a level expected for a seed st rage protein, and the beta-zein clone pZG15RX (Pedersen et al., ibid). The phaseolin promoter was made accessible by a site specific mutation at position -7 which resulted in a BglII site, thus the phaseolin promoter could be removed after a BglII digest as an 800 bp fragment. This fragment was subcloned into the BamHI site of pUC18 (available from commercial sources), yielding a plasmid designate pUC-Pvpro. The beta-zein structural gene, including signal peptide, coding region, and Poly (A) addition signal was removed from plasmid pZG15EX (available from B. Larkins, Purdue University, West Lafayette, IN) after a TagI digestion and this fragment was cloned into the AccI site of pUC-Pvpro, yielding clone pUC-Phas-zein. This Phas-zein gene was removed by digestion with HindIII and EcoRI and this fragment was cloned into the binary vector pGA482G, which had previously been digested with HindIII and EcoRI. This new binary plasmid is referred to as pGA482G-Phas-zein (see Chart 2) and it was transferred into Agrobacterium strains: A208, C58, LBA4404, C58Z707, and A4RS which in turn can be used to produce transformed plants in accordance with the method of this invention.

A phase zein construction similar to that described above has been transferred into dicotyledonous plants and its developmental expression in the seeds of the transformed plant has been obs rv d; s Hoffman t al. (1987) EMBO J. 6:3213. Additional modificati n has been made t a Phas-zein gene c nstruction. Th s m difications include th ligation f a BglII linker nt its 5'- nd and a BamHI link r onto its 3'- nd which allows th constructi n of multiple c pies of th phase zein gene as described above for the phase lin minig ne. This allows for the transfer f a HSSP-gene multigene

family into a plant species by a single transformation event and the expression of higher levels of the HSSP-gene product. This leads to the development of dicotyledonous plant varieties which are nutritionally improved, such as common bean, soybean and other large seeded plants.

Example 4 Transfer of Viral Resistance

The purpose of this example is to generate a construction for the expression of a plant virus coat protein gene which, when expressed in a dicotyledonous plant, results in reduced symptoms or resistance to later infections by that virus (see report by Powell-Abel et al. (1986) Science 232:738). Viral coat proteins are isolated from any number of plant virus classes (tobamo, cucum, poty, tobra, AMV, etc.) and they are expressed constitutively in plants after the attachment of the CaMV 35S promoter. In addition, a plant poly (A) signal is added to the 3' region to ensure proper expression.

A clone containing any specific viral coat protein gene can be obtained for both plant DNA and RNA viruses. Such is the case for cucumber mosaic virus strain C (CMV-C); its RNA genome was copied into double-stranded cDNA and the coat protein gene was isolated and characterized as follows. A residues were added to the 3' end of CMV-C total RaH, using E. coli polyadenylose. This poly (A) region was used to anneal an aligo dT primer which was used to prime the synthesis of single-stranded (SS) cDNA using reverse transriptos and appropriate buffer of CMV-C SS-cDNA, double-stranded cDNA was synthesized by adding RNaso H to remove the RNA from the duplex and the second strand was made by adding E. coli DNP polymerase I (Kl now fragment) and the appropriate buffer. After synthesis of CMA-C ds-DNA, it was E. coli methylated using Eco RI methylase and Eco methylent buffer, thus protecting all internal Eco RI sites in the CMV-C ds-cDNA molecules. After Eco methylation the CMV-C ds-cDNA molecules were treated again with E coli polymorse I (Klenow fragment) to ensure that all ends (5' and 3') were flush, then these molecules were ligated to Eco RI linkers using T4-Ligase. After ligation the CMV-C ds-cDNA molecules were separated from contaminating linker by size fractionation on a GYOG column (1cm X 30cm). The fraction containing the majority of the CMV-C ds-cDNA molecules was EtOH precipitated, followed by resuspension in 10 μg of H20.

About 100 μg of these EcoRI linked CMV-C ds-cDNA molecules were removed and mixed with 1μg of λ gT11 arms (commercially available) and ligated together using T4 ligase. The recombinant GT 11-CMV-C were plated using E coli Up50supF as host and these plates (10^{-4} clones) were screened for clones containing CMV-C coat protein gen coding region using p-labeled CMV-whiteleaf SS-cDNA as probe. From this screening, a clone, λ GT11-CMV9.9 was isolated. It contained an EcoRI insert of 1400 base pair, enough to encode the complete CMV coat protein. This CMV coat protein gene can be expressed in plant tissues once a plant-active promoter and poly (A) signal are attached to its 5' and 3' regions, respectively. The scheme to accomplish this is shown in Chart 3.

Attachment of the constitutive cauliflower mosaic virus (CaMV) 35S promoter was done by first doing a partial AccI and complet EcoRI digests of clone pCMV9.9 which was obtained by cloning the Eco RI insert from Lambda GT11-CMV9.9 into EcoRI cut puc 19 (commercially available). The 1100 bp CMV-C coat protein gene fragment was removed, both ends were blunted, and this fragment was cloned int the SmaI site of pDH51 (Pietrzak et al. (1986). Nuc. Acids Res. 14:5857) which is available from A.T. Mohn, Friedrick Mieschen Institut, Basel, Switzerland to obtain clone pDH51/cP19.. This positioned the CMV-C coat protein gene downstream of the CaMV 35S promoter and upstream from the CaMV poly (A) signal sequence. T ensure a high level of expression other poly (A) signal sequences (which may function better than the CaMV 35S poly (A) signal) can b attached, such as the poly (A) signal from the seed storage protein gene phaseolin (Slightom et al. (1983) Proc. Natl. Acad. Sci. 80:1897). To facilitate engineering, this plant expressible CMV-C coat protein gene was removed from clone pDH51/CP19 by an EcoRI digest and the 1800 bp fragment was cloned into pUC1813 (which contains more restriction enzyme sites and is available from Dr. R. Kay, Washington State University, Pullman, Washington. The resulting 1 ne, pUC1813/CP19, was th n partially digested with HindIII and the 1800 bp fragment was cloned int th binary vector pGA482 t btain th n w cl ne, pGA482/CP19H (s Chart 3). This binary plasmid, or its derivatives, can b transf rr d into Agrobacterium strains: A208, C58, LBA4404, C58Z707, A4RS, A4RS(pRiB28b) and th rs. Using th transformation m thod f this inventi n, this plant xpr ssible CMV-C

coat protein gene (or any other plant virus coat protein gene) can be transferred into a dicotyledonous plant species such as, cucumber, squash, melon, zucchini, pepper, etc. The development of these new cultivars are useful because of their resistance to infections by specific virus or viruses (if more than one virus coat protein gene construction is transferred to a single plant).

Example 5 Transfer of Herbicide Resistance

The purpose of this example is to illustrate how to generate plant expressible genes which allow a plant to be resistant to specific classes of herbicides. Such plants are useful for many reasons; (i) herbicides normally lethal can be used, and (ii) different crops can be used in close rotations on soil which may contain residual amounts of a previously used herbicide that is normally lethal to the second crop. Two genes of interest are mutant derivatives (derived from plant or bacterial sources) of the acetolactate synthase (ALS) gene which are not sensistive to chlorsulfuron and sulfometuron methyl herbicides (Falco et al., (1985) Biotech. Plant Sci. Academic Press, Inc. page 313) and mutants of the gene encoding enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Stalker et al, (1985) J. Biol. Chem., 260:4724) which are not sensitive to the herbicide glyphosate.

A gene which encodes an important enzyme which is either resistant to or detoxifies a specific herbicide is cloned downstream from a plant active promoter, such as: CaMV 35S, ribulose-1,5-bisphosphate carboxylase small subunit gene, or other strong plant gene promoter and upstream from a plant gene poly (A) signal sequence, see Chart 4.

This gene is then be cloned into an Agrobacterium-derived vector (either binary or cis) and using the above-described plant transformation method, such a gene is be transferred into many dicotyledonous plant species, such as: soybean, common bean, peppers, melons, etc.

Example 6 Transfer of Insect-Resistant Gene

In nature, numerous polypeptides exist which are toxic to insect pests. The best known protein toxins are those associated with different strains of Bacillus thuringiensis; for example, B. israelenis activ against Diptera (mosquitos and blackflies), B. thuringinensis activ against Lepidoptera, and B. san diego active against Coleoptera. The toxi protein found in each of these bacteria is highly specific to insect pests; they are not toxic to other

organisms. Thus the transfer and expression of genes encoding such toxic proteins in plants are beneficial in reducing insect damage without using chemical insecticides thereby avoiding risk to other organisms. The genes encoding many of these toxic proteins have been isolated and sequenced (Schnepf et al. (1985) J. Biol. Chem., 260:6264; Waalwijk et al., (1985) Nucl. Acids Res., 13:8207; Sekar et al (1987) Proc. Natl. Acad. Sci., 84:7036). The transfer of the B.thuringiensis toxic gene into tobacco and its usefulness in protecting the plant from insect damage has been reported (Vaeck et al. (1987) Nature 328:33). Thus, the combination of using the plant transformation system described here and plant expressible Bacillus toxin gene (see Chart 5) allows for the transfer of a useful trait to any dicotyledonous species for which tissue-culture based transformation systems are inefficient or have not been developed, such as: common bean, soybean, melon, cucumber, squash, zucchini, pepper, etc.

Chart 1

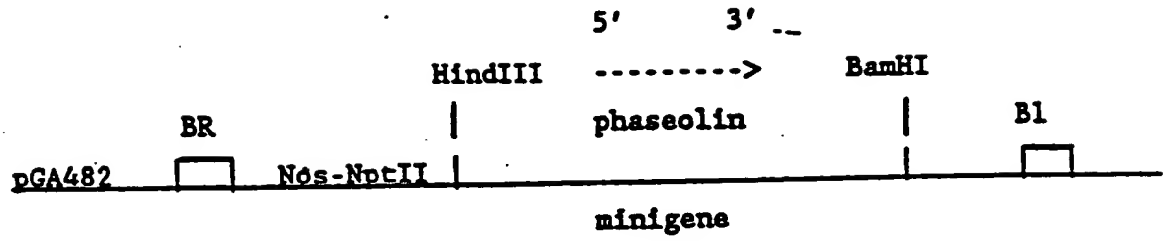


Chart 2

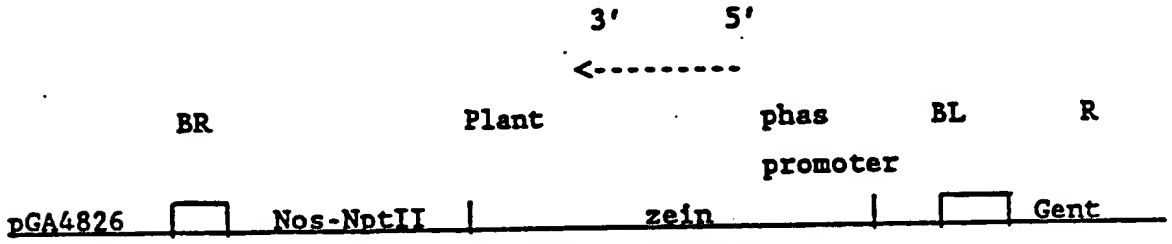


Chart 3

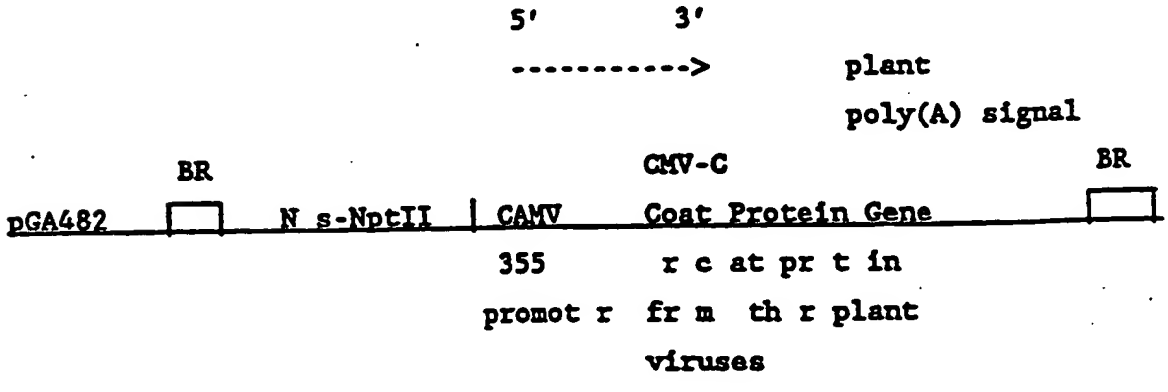


Chart 4

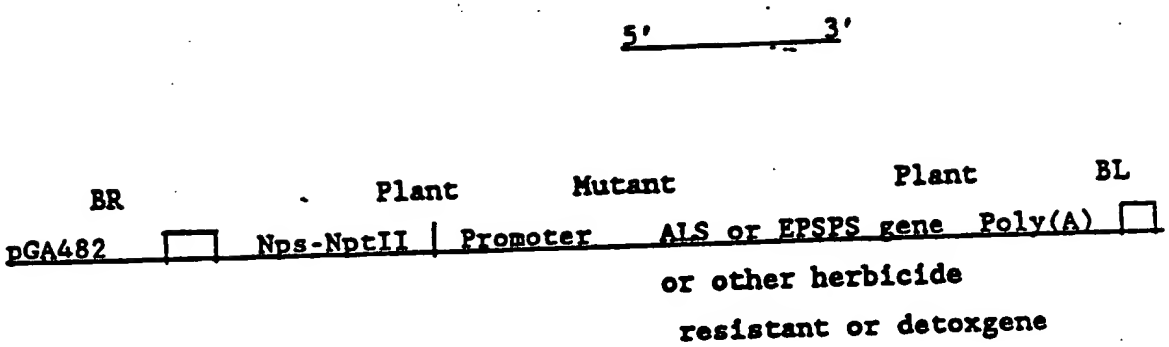
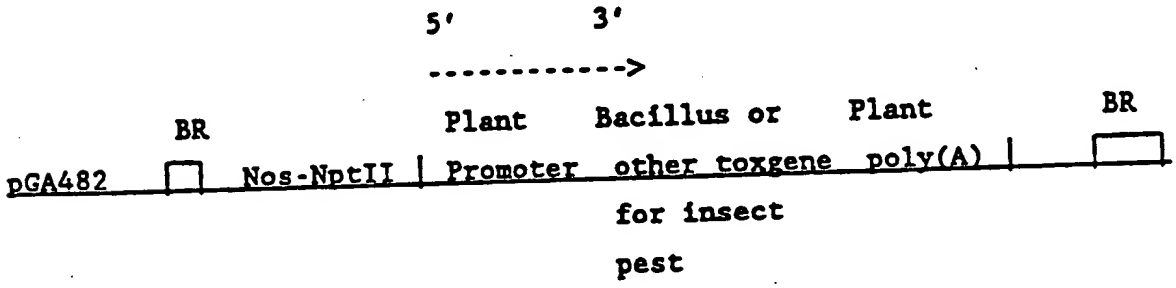


Chart 5



CLAIMS

1. A process for producing a transgenic plant which comprises:

(a) germinating a seed of a plant;

(b) inoculating the meristematic or mesocotyl cells produced during germination, prior to their differentiation, with a virulent or non-virulent Agrobacterium strain containing a transferable gene in an Agrobacterium derived vector; and

(c) allowing the cells to differentiate into mature plants, with the proviso that the plant cannot be from the family Arabidopsis thaliana.

2. A process according to claim 1 wherein the vector is a plasmid adapted for either transfer in trans- or cis- configuration.

3. A process according to claim 2 wherein the the vector is a binary plasmid adapted for transfer in the trans configuration.

4. A process according to claim 3 wherein the plant is dicotyledonous.

5. A process according to claim 4 wherein one of the cotyledons is removed prior to inoculation.

6. A transgenic plant prepared by the process of claim 1.

7. A transgenic dicotyledonous plant according to claim 6.

8. A plant according to claim 7 wherein the plant is a member of the family leguminoseae.

9. A plant according to claim 8 wherein the plant is a soybean.

10. A plant according to claim 9 wherein the plant is common bean.

11. A plant according to claim 10 wherein the plant gene is the gene for phaseolin.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/04464

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00; A 01 H 1/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N; A 01 H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	FR, A, 2560744 (PHYTOGEN) 13 September 1985, see the whole document --	1 1-10
	Mol.Gen Genet, vol. 208, no. 1/2, June 1987, Spring-Verlag K.A. Feldmann et al.: "Agrobacterium-mediated transformation of germinating seeds of Arabidopsis thaliana: A non-tissue culture approach", pages 1-9 see the whole document --	1-10
X	Plant Mol. Biol., vol. 8, no. 3, 1987, M. Nijhoff Publishers, Dordrecht (NL) K.Sukhapinda et al.: "Ri-plasmid as a helper for introducing vector DNA into alfalfa plants", pages 209-216 see abstract --	6-8
O,X	Biological Abstracts/RRM, no. 89:116856 T.C. Hall et al.: "Transformation of plant cells", see abstract 36:62272 & Ciba Foundation Symposium, no. 137, Applications of plant cell and tissue	6-8,10

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th April 1989	27. 05. 89
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

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	culture; symposium, Kyoto, Japan, 20-22 October 1987, IX+269P. John Wiley and Sons, Inc.: Somerset, NJ US, Chistester, GB Illus. 0 (0), 1988, 123-138	
O,X	Biological Abstracts/RRM W. Lin et al.: "Soybean tissue culture and genetic transformation" see abstract 33117694 & Int Bot Congr Abstr 1987. vol. 17, no. 0, p167, --	6-9
O,X	J. Cell Biochem. Suppl. 11B, S.L. Goldman et al.: "Transformation of Zea mays by Agrobacterium tumefaciens: Evidence for stable genetic alterations", page 26, see abstract F 202 --	1,2,6
P,X	Chemical Abstracts, vol. 109, 1988, (Columbus, Ohio, US) see page 193, abstract 105884p & JP, A, 6387921 (UNIVERSITY OF TOLEDO) 19 April 1988 --	1-3,6
P,X	EP, A, 0267159 (CIBA-GEIGY & LUBRIZOL GENETICS) 11 May 1988, see claims --	1-10
P,X	EP, A, 0256751 (LUBRIZOL GENETICS) 24 February 1988, see the whole document --	6-9
A	EP, A, 0241963 (C.E.N.) 21 October 1987, see the whole document --	1-10
A	Plant. Molecular Biology, vol. 7, 1986 M. Nijfhoff Publishers, Dordrecht (NL) A.C.F. Graves et al.: "The transformation of Zea mays seedlings with Agrobacterium tumefaciens" pages 43-50 see the whole document --	1-10
A	Nature, vol. 325, no. 7000, 7-14 January 1987, (Neptune, NJ, US) N. Grimsley et al.: "Agrobacterium-mediated delivery of infectious maize streak virus into maize plants", pages 177-179, see the whole document --	1-10

III. DOCUMENTS CONSIDERED T BE RELEVANT (C NTINUED FR M THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	EP, A, 0064720 (RESEARCH AND DEVELOPMENT INSTITUTE INC. MONTANA) 17 November 1982, see exemple 13; table 13 --	10
E	EP, A, 0301749 (AGRACETUS) 1 February 1989, see example 8	6-9

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8804464 SA 25950

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent ffice EDP file on 16/05/89
The European Patent Office is in no way liabl for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
FR-A- 2560744	13-09-85	None		
EP-A- 0267159	11-05-88	AU-A-	8089387	12-05-88
		JP-A-	63141590	14-06-88
EP-A- 0256751	24-02-88	AU-A-	7654187	11-02-88
		JP-A-	63177795	21-07-88
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		AU-B-	537644	05-07-84
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